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### Title

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### Permalink

<https://escholarship.org/uc/item/99x488s2>

### Journal

American Journal of Obstetrics and Gynecology, 216(3)

### ISSN

0002-9378

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### Publication Date

2017-03-01

### DOI

10.1016/j.ajog.2016.08.041

Peer reviewed



Published in final edited form as:

*Am J Obstet Gynecol.* 2017 March ; 216(3): 290.e1–290.e9. doi:10.1016/j.ajog.2016.08.041.

## Maternal Bisphenol A Exposure Alters Rat Offspring Hepatic and Skeletal Muscle Insulin Signaling Protein Abundance

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### Abstract

**Background**—The obesogenic and diabetogenic effects of the environmental toxin BPA during critical windows of development are well-recognized. Liver and skeletal muscle play a central role in the control of glucose production, utilization and storage.

**Objectives**—We hypothesized that maternal BPA exposure disrupts insulin signaling in rat offspring liver and skeletal muscle. We determined the protein expression of hepatic and skeletal muscle insulin signaling molecules including insulin receptor beta (IR- $\beta$ ), its downstream target insulin receptor substrate 1 (IRS-1) and glucose transporters (GLUT2; GLUT4) and hepatic glucose regulating enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucokinase (GCK).

**Study Design**—Rat dams had *ad libitum* access to filtered drinking water (Control) or drinking water with BPA from 2 weeks prior to mating and through pregnancy and lactation. Offspring litters were standardized to 4 males and 4 females and nursed by the same dam. At weaning, BPA exposure was removed from all offspring. Glucose tolerance was tested at 6 weeks and 6 months. Liver and skeletal muscle was collected from 3 week-old and 10 month-old offspring for protein expression (Western Blot) of IR- $\beta$ , IRS-1, GLUT2, GLUT4, PEPCK and GCK.

**Results**—Male, but not female BPA offspring had impaired glucose tolerance at 6 weeks and 6 months. Both male and female adult offspring had higher glucose-stimulated insulin secretion as well as ratio of stimulated insulin to glucose. Male BPA offspring had higher liver protein abundance of the 200 kD IR- $\beta$  precursor (2-fold), and IRS-1 (1.5-fold), while GLUT2 was 0.5-fold of the control at 3 weeks of age. In adult male BPA offspring the abundance of IR- $\beta$  was

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**Conflicts:** The authors report no conflict of interest

**Paper presentation information:** SMFM's 36th Annual Pregnancy Meeting, Atlanta GA, February 1–6, 2016

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higher (2-fold) and GLUT4 was 0.8-fold of the control in skeletal muscle. In adult female BPA offspring the skeletal muscle protein abundance of GLUT4 was 0.4–fold of the control.

**Conclusions**—Maternal BPA had sex- and tissue-specific effects on insulin signaling components, which may contribute to increased risk of glucose intolerance in offspring. Glucose transporters were consistently altered at both ages as well as in both sexes and may contribute to glucose intolerance. These data suggest that maternal BPA exposure should be limited during pregnancy and lactation.

### Keywords

BPA; fetal programming; GLUT4; Insulin Signaling; Rat

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## INTRODUCTION

The world-wide incidence of metabolic diseases, including type II diabetes, has increased dramatically over the past 30 years.<sup>1,2</sup> While there is little doubt that diet, exercise and genetic factors all play a role in an individual's susceptibility to type II diabetes, evidence from human and animal models suggests that predisposition to the development of metabolic disease also begins *in utero*.<sup>3</sup> This concept of “developmental programming” states that early environmental exposures during pregnancy and/or lactation programs changes in gene expression that alters growth and development with consequences for the long-term health of the offspring.<sup>3</sup>

Recent public health concerns have been raised regarding the potential long-term effects of exposure to the endocrine disrupter chemical bisphenol A (4,4-dihydroxyl-2,2-diphenylpropane; BPA) during the vulnerable period of perinatal development.<sup>4</sup> BPA is an industrial chemical used primarily to make polycarbonate plastic and epoxy resins. It is used in the production of everyday items including carbon-free paper, sports equipment, medical devices, reusable food and drink containers and dental sealants. The abundant use of BPA has made this endocrine disrupter chemical ubiquitous in our environment, leading to chronic low dose exposure.<sup>5</sup> According to the 2003–2004 National Health and Nutrition Examination Survey (NHANES) BPA was detected in the urine of >90% of the survey participants.<sup>6</sup> Moreover, children had the highest urinary BPA concentrations, followed by adolescents and adults.<sup>6</sup> In addition, BPA has been detected in maternal serum, amniotic fluid, fetal cord blood and breast milk.<sup>7</sup>

During periods of increased glucose availability, tight homeostatic regulation of blood glucose levels is primarily achieved by the actions of insulin to inhibit hepatic glucose production and to increase the uptake and storage of glucose in peripheral insulin-sensitive tissues, such as skeletal muscle.<sup>8</sup> Hence, any disruption to glucose-stimulated insulin secretion or to the ability of peripheral tissues to respond to insulin action via intracellular insulin signaling pathways will likely have adverse consequences for glucoregulation.<sup>8</sup> Using NHANES data (2003–2004; 2005–2006; 2007–2008), the majority of published human epidemiological studies have shown positive associations between urinary concentrations of BPA, glucose intolerance and diabetes.<sup>9–12</sup> Similarly, studies in animals have shown impaired glucose tolerance and altered secretion of insulin from the endocrine

pancreas in adult mice<sup>13–16</sup> and rats<sup>17;18</sup> that were exposed to BPA perinatally. Studies in adult mice and rats have implicated changes in pancreatic  $\beta$ -cell mass, as well as lower expression of genes that normally optimize  $\beta$ -cell function, such as *Pdx-1*, in the mechanism by which BPA alters glucoregulation.<sup>14;17;20;21</sup> However, homeostatic regulation of blood glucose is determined by both pancreatic  $\beta$ -cell insulin secretion and its effects on peripheral, insulin-sensitive tissues. The biological effects of insulin, including uptake of glucose into fat and muscle cells and suppression of glucose synthesis in the liver are mediated by activation of the insulin receptor and the biochemical insulin transduction pathway. Previous studies have focused on BPA-induced alterations in insulin secretion and beta cell mass or function<sup>14;17;20;21</sup>, but whether perinatal exposure to BPA has age-, sex- or tissue-specific effects on the protein abundance of insulin signaling components is unknown. Hence, the current study determined whether perinatal exposure to BPA alters the protein abundance of insulin signaling components in offspring liver and muscle at weaning and in adulthood.

## MATERIALS AND METHODS

All procedures were approved by the Animal Care Committee at the Los Angeles Biomedical Research Institute at Harbor-University of California, Los Angeles, and were conducted in accordance with guidelines provided by the American Accreditation Association of Laboratory Care and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

### Animals

Eleven virgin female Sprague Dawley rats (9 weeks old; Charles River Laboratories, Hollister, CA) were housed in a facility with constant temperature ( $21 \pm 1$  °C) and a controlled 12:12-hour light/dark cycle. A rat model of maternal exposure to BPA was created using *ad libitum* access to BPA (Sigma-Aldrich Corp., St. Louis, MO, USA) in drinking water 2 weeks prior to and through pregnancy and lactation. The amount of BPA consumed was  $239 \pm 8$   $\mu\text{g/day/body weight}$  over the course of pregnancy and  $466 \pm 33$   $\mu\text{g/day/body weight}$  during lactation (due to increased water intake during lactation). At birth, blood from all excess newborn pups was pooled to create sufficient volume to determine the plasma BPA level. Newborns from BPA-treated dams had a plasma BPA level (0.62 ng/ml) within the range of values measured in human umbilical cord blood, whereas BPA was undetectable in plasma from the newborns of Control dams.<sup>19;20</sup> There were no differences in body weights between control (male:  $6.85 \pm 0.10$  g; female:  $6.62 \pm 0.11$  g) and BPA-treated (male:  $6.84 \pm 0.18$  g; female:  $6.65 \pm 0.08$  g; two way ANOVA with sex and treatment as factors) offspring within each sex at birth, or at any time point thereafter.

At 11 weeks of age, rats were mated and continued on their respective treatments during pregnancy and lactation. After birth, at 1 day of age, litters were culled to 8 pups (4 males and 4 females) per dam to standardize nursing. All pups were nursed by their respective dams until 3 weeks of age. At weaning BPA exposure was removed and the rat pups were housed 4 per cage with the same sex. The animals were separated to two per cage at 125 g and into singular housing when their weight was above 250 g, such that no cage contained

more than 500 g of total rat body weight. All rats were housed in BPA-free polycarbonate cages (Ankar Corp; Bellmore, NY). The cages were filled with paperchip bedding (Sherpherd Specialty Papers; Watertown, TN) with cardboard tubes for enrichment. All weaned offspring had *ad libitum* access to a standard control diet (LabDiet 5001; LabDiet, St. Louis, MO) and filtered drinking water with no further BPA exposure.

### Glucose tolerance and plasma analyses

At 6 and 24 weeks of age, 1 male and 1 female offspring from each litter underwent a glucose tolerance test (GTT). Following an overnight fast, D-glucose (1 mg/g body weight) was injected intraperitoneally (i.p) in conscious rats. Blood glucose values were determined in tail bleed blood prior to (time 0) and 15, 30, 60, 120, and 180 minutes after glucose administration using a Hemocue B-glucose analyzer (HemoCue Inc, Mission Viejo, CA). Plasma insulin concentrations were determined prior to the glucose challenge (time 0; 6 and 24 weeks of age) and during the challenge at 15 and 180 minutes (24 weeks). Blood was collected into heparinized tubes at 0, 15 and 180 minutes, centrifuged immediately at 3,000 g and 4°C for 10 min and the plasma was stored at -80 °C. Plasma insulin concentrations were measured using a commercially available, rodent-specific ELISA kit (10-1250-01; Mercodia, Uppsala, Sweden).

### Tissue collection

At 3 weeks and 10 months of age, offspring were anesthetized by 5% isoflurane/2% oxygen by mask and exsanguinated via cardiac puncture. Euthanasia was confirmed by decapitation. Plasma, as well as liver and gastrocnemius skeletal muscle tissue were collected, snap-frozen in liquid nitrogen and stored at -80 °C until analysis. The offspring in the current study were part of a larger study of the long-term effects of perinatal BPA exposure on offspring growth and appetite regulation (these data will be published separately). Consequently, offspring tissues were not available at the exact ages corresponding to the *in vivo* measurements of glucose clearance. Tissues collected at ages closest to the *in vivo* experiments were used to determine the protein abundances of components of the insulin signaling pathway.

### Protein extraction and Western blotting

Liver and skeletal muscle protein was extracted in radioimmunoprecipitation assay buffer (1x) containing protease inhibitors (HALT cocktail; Pierce, Rockford, IL) and the total protein concentration in the supernatant was determined using a bicinchoninic acid assay (BCA kit; Pierce, Rockford, IL). Western Blotting was performed as previously described,<sup>21</sup> using primary antibodies from Santa Cruz Biotechnology (Texas, USA; 1:1000) against insulin receptor beta subunit (IR-β; sc-711), insulin receptor substrate 1 (IRS-1; sc-559), glucose transporter 2 (GLUT2; sc-9117), glucose transporter 4 (GLUT4; sc-1607), phosphoenolpyruvate carboxykinase (PEPCK; sc-32879) and glucokinase (GCK; sc-7908). Goat anti-rabbit IgG HRP conjugate (#1706515; Bio-Rad, California, USA; 1:2000) and donkey anti-goat IgG HRP conjugate (sc-2020; Santa Cruz Biotechnology, Texas, USA; 1:2000) were used as secondary antibodies. The depicted bands had the expected molecular weights.

## Statistical analysis

For all studies 5–6 offspring were studied per group (one of the 4 male offspring and one of the 4 female offspring from each of 6 control and 5 BPA-treated litters), with the exception of newborn plasma for BPA measurement where blood was pooled from all excess pups. No differences were found in the protein abundance of insulin signaling components in skeletal muscle from the 6 week-old glucose intolerant male offspring, hence skeletal muscle was not analyzed from 6 week-old female offspring that did not show a glucose intolerant phenotype at 3 weeks of age. Values are means  $\pm$  standard error of the mean (SEM). Statistical significance (SigmaStat Statistical Software version 2.0) was assessed using unpaired Student's t- test and two-way repeated measures ANOVA, as appropriate. Statistical significance was accepted when  $P < 0.05$ .

## RESULTS

### Fasting glucose and insulin

At 3 and 6 weeks of age, male and female fasting blood glucose and plasma insulin concentrations, and the ratio of insulin to glucose, were not different between control and BPA offspring. In contrast, at 24 weeks of age, male BPA offspring had higher plasma insulin concentrations, though the blood glucose concentrations and the ratio of insulin to glucose were comparable to controls. In addition, 24 week-old female BPA offspring had similar blood glucose concentrations and insulin levels, though the ratio of insulin to glucose was increased compared to controls (Table 1).

### Glucose tolerance test (GTT)

At both ages (6 and 24 weeks), administration of glucose generated an immediate increase in the blood concentration of glucose ( $P < 0.05$ ) that was maximal between 15 and 30 minutes post-injection in both BPA and control offspring (Figure 1). In 6 week-old BPA males, the area under the glucose curve was higher than in controls and remained higher in adulthood at 24 weeks of age (Figure 1A and 1C). However, perinatal exposure to BPA did not affect the time course of glucose clearance or the area under the glucose curve in female offspring at 6 or 24 weeks of age (Figure 1B and 1D).

At 24 weeks of age, the increase in blood glucose was accompanied by rapid insulin release in all offspring. Although there were similar rates of glucose clearance in BPA and control male and female offspring, the area under the glucose-stimulated insulin curve was higher in both BPA males and females compared with controls (Figures 1C and 1D). Additionally, the ratio of stimulated insulin to glucose was higher in both male and female BPA offspring (Table 1).

### Liver Insulin signaling components

At 3 weeks of age, the protein abundance of GLUT2 was lower and the abundances of the 200 kD IR- $\beta$  precursor and IRS-1 were higher in male BPA offspring compared with controls (Figure 2A). However, perinatal BPA did not affect any of the liver insulin signaling components in female BPA offspring (Figure 2C and 2D). Furthermore, the protein

abundance of GCK or PEPCK liver enzymes was not different in either sex (Figure 2A – 2D).

At 10 months of age, there were no differences in the protein abundance of any of the measured liver insulin signaling components or enzymes in BPA male or female offspring (Figures 2B and 2D).

### Skeletal Muscle Insulin signaling components

At 3 weeks of age, the protein abundance of GLUT4 and IR- $\beta$  was not different between male BPA and control offspring (Figure 3A). In contrast, at 10 months of age, the protein abundance of GLUT4 was lower in both BPA male and female offspring. In addition, BPA males had higher abundance of IR- $\beta$  compared with controls (Figures 3B and 3C).

## COMMENT

Perinatal exposure to BPA was associated with age- and sex-dependent changes in glucose tolerance and insulin sensitivity, consistent with previous rodent studies.<sup>14;17;22;23</sup> Since blood glucose regulation is determined by both insulin secretion and peripheral insulin sensitivity we determined the effects of perinatal BPA on the protein abundance of components of the insulin signaling pathways in offspring liver and skeletal muscle. The present study showed lower protein abundance of GLUT2 and higher protein abundance of IRS-1 in the liver of male BPA-treated offspring at weaning. In addition, both BPA male and female offspring had lower protein abundance of GLUT4 in skeletal muscle in adulthood.

Higher abundance of the insulin receptor precursor molecule in male offspring liver at weaning, as well as in skeletal muscle in adulthood, was not associated with age-, sex-, or tissue-specific changes in the protein abundance of the mature, functional IR- $\beta$  subunit. These data contrast with decreased abundance of the mature IR- $\beta$  subunit in liver and skeletal muscle from adult rats after exposure to a high dose (200 mg/kg body weight) of BPA.<sup>24,25</sup> Conversely, the protein abundance of IRS-1 was higher in the liver of male BPA-treated offspring, consistent with BPA-increased IRS-1 in adult mouse liver.<sup>26</sup> In the latter study, the authors speculated that higher liver protein abundance of IRS-1 in BPA than control mice may counteract the BPA-induced decrease in insulin receptor phosphorylation as a compensatory mechanism to avoid insulin resistance.<sup>26</sup> Whether BPA altered insulin receptor phosphorylation in the current study is unknown, since that the data are limited by the collection of offspring tissues after an overnight fast and not under insulin-stimulated conditions.

At weaning, the protein abundance of the bi-directional glucose transporter GLUT2 was lower in liver tissue from BPA- than from control-males. Similarly, lower GLUT2 abundance has been demonstrated in isolated pancreatic islets *in vitro*,<sup>27</sup> in adult rat testis and pancreas *in vivo*,<sup>28</sup> and in the pancreas of male and female offspring after BPA treatment.<sup>17</sup> In contrast, studies of adult male mice and rats exposed to BPA demonstrated variable, dose-dependent effects on GLUT2 expression in the liver.<sup>24;29</sup> Notably, lower liver GLUT2 abundance in the current study did not likely impair liver maintenance of blood



glucose levels in the fasted state, since there were no differences in fasting blood glucose levels between control and BPA males at 3 weeks of age.

Lower abundance of the glucose transporter GLUT4 in skeletal muscle from adult male and female offspring is consistent with previous studies of BPA-treated adult male rats.<sup>25</sup> Since skeletal muscle is the major peripheral site of glucose uptake, usage and storage, any BPA-induced deficiency in skeletal muscle insulin signaling is likely to predispose offspring to the development of insulin resistance.<sup>30</sup> Moreover, heterozygous GLUT4 knockout mice exhibit peripheral insulin resistance, specifically implicating the GLUT4 transporter in the development of insulin resistance.<sup>31–33</sup> Accordingly, in the current study both male and female adult offspring were insulin resistant, as evidenced by higher insulin:glucose ratios and greater area under the insulin curves during the glucose tolerance test. However, since tissue for protein studies was not collected at the time of the glucose tolerance test further mechanistic studies are required to confirm that lower GLUT4 abundance contributes to BPA-induced glucose intolerance and to determine whether perinatal BPA affects translocation of the GLUT4 transporter to the plasma membrane and subsequent glucose uptake.<sup>25</sup>

Perinatal exposure to BPA induces sex-specific changes in rat liver lipid metabolism<sup>34</sup> and disrupts sexually dimorphic behaviors in mice.<sup>35</sup> The molecular mechanism underlying the sex-specific effects of BPA in these studies and in the current study is unknown, but may be mediated by BPA interactions with estrogen receptors and estrogen signaling pathways. BPA can bind to both classical and non-classical G-protein-coupled estrogen receptors<sup>36–38</sup> that regulate central and peripheral glucose metabolism<sup>39</sup> and may interfere with estrogen signaling by activation or inhibition of the receptor.<sup>40</sup> Classical estrogen receptors show sex- and tissue-specific patterns of expression throughout development, but whether these signaling pathways are disrupted during perinatal development in the insulin sensitive tissues of BPA-exposed offspring is unknown. BPA may also alter insulin signaling by altered methylation of genes, such as GLUT4.<sup>41</sup> Indeed, altered methylation of the estrogen receptor  $\alpha$  (*Esr1*) and several other genes has been shown following perinatal exposure to BPA.<sup>35;42</sup> Gene methylation and demethylation is catalyzed by DNA methyltransferase (DNMT) enzymes. Accordingly, altered expression of the enzymes DNMT1 and DNMT3a have been demonstrated in rodent brain tissue<sup>35</sup> and in preadipocytes exposed to BPA *in vivo* and *in vitro*, respectively.<sup>19</sup> Further studies of receptor expression patterns and site-specific gene methylation are required to correlate epigenetic effects of BPA, sex- and tissue-specific changes in gene expression and offspring phenotypes. In addition, although there were no differences in weights between control and BPA offspring in the current study, changes in the insulin signaling pathway may have occurred secondary to BPA-induced changes in offspring body composition (fat mass), lipid homeostasis and/or inflammation.<sup>19;34</sup>

Using a model of maternal treatment with BPA that produces maternal and newborn plasma BPA concentrations that are similar to the range of values measured in serum from pregnant women and human umbilical cord blood<sup>19</sup> we have shown that BPA disrupts glucose homeostasis and alters the abundance of insulin signaling components in the exposed



offspring. Early life exposure to BPA should therefore be considered a possible risk factor for the development of insulin resistance and diabetes.

## Acknowledgments

**Financial Support:** This work was supported by the National Institutes of Health Grants R21ES023112-01 (MD, MGR) and RO3 HD079722-01 (JKJ, MD, MGR), and National Center for Advancing Translational Sciences UCLA- CTSI Grant U11TR000124 (MD).

The authors thank Stacy Behare for technical assistance.

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Figure 1A) 6 Weeks: Male Glucose Tolerance Test

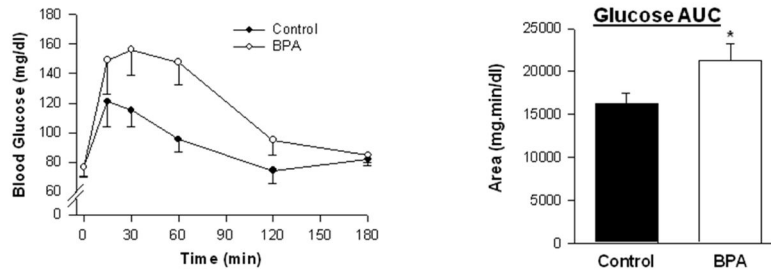


Figure 1B) 6 Weeks: Female Glucose Tolerance Test

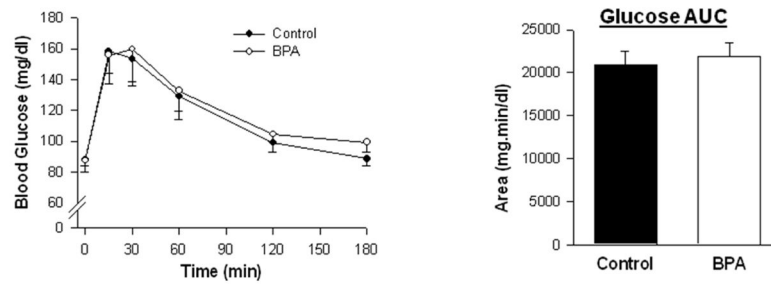
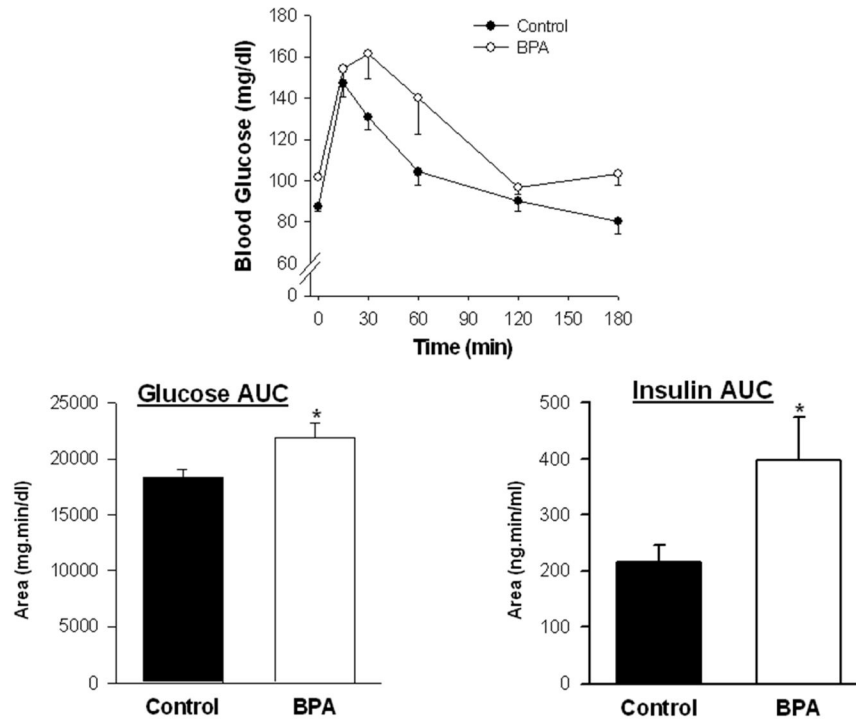
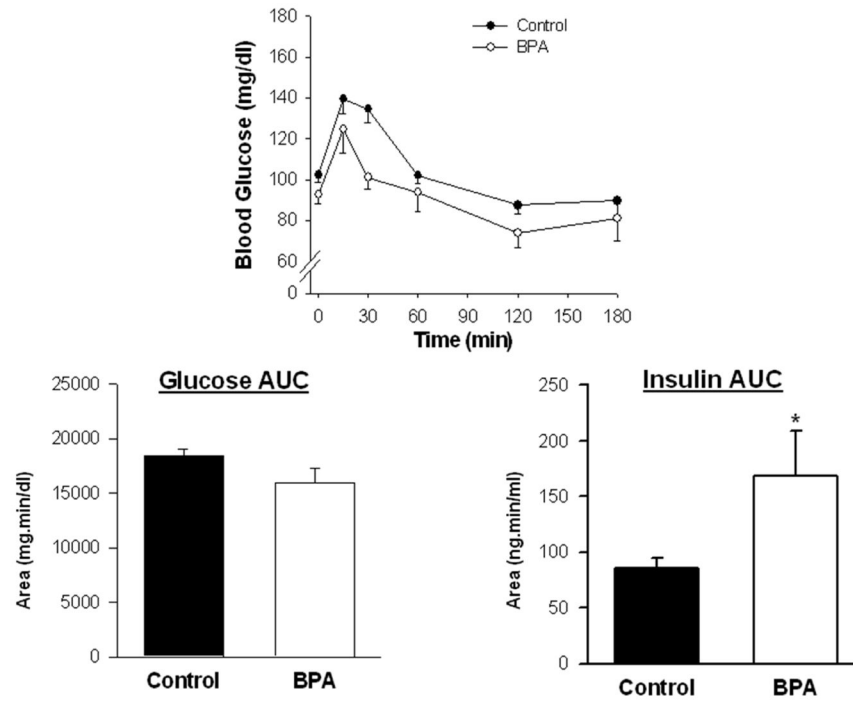
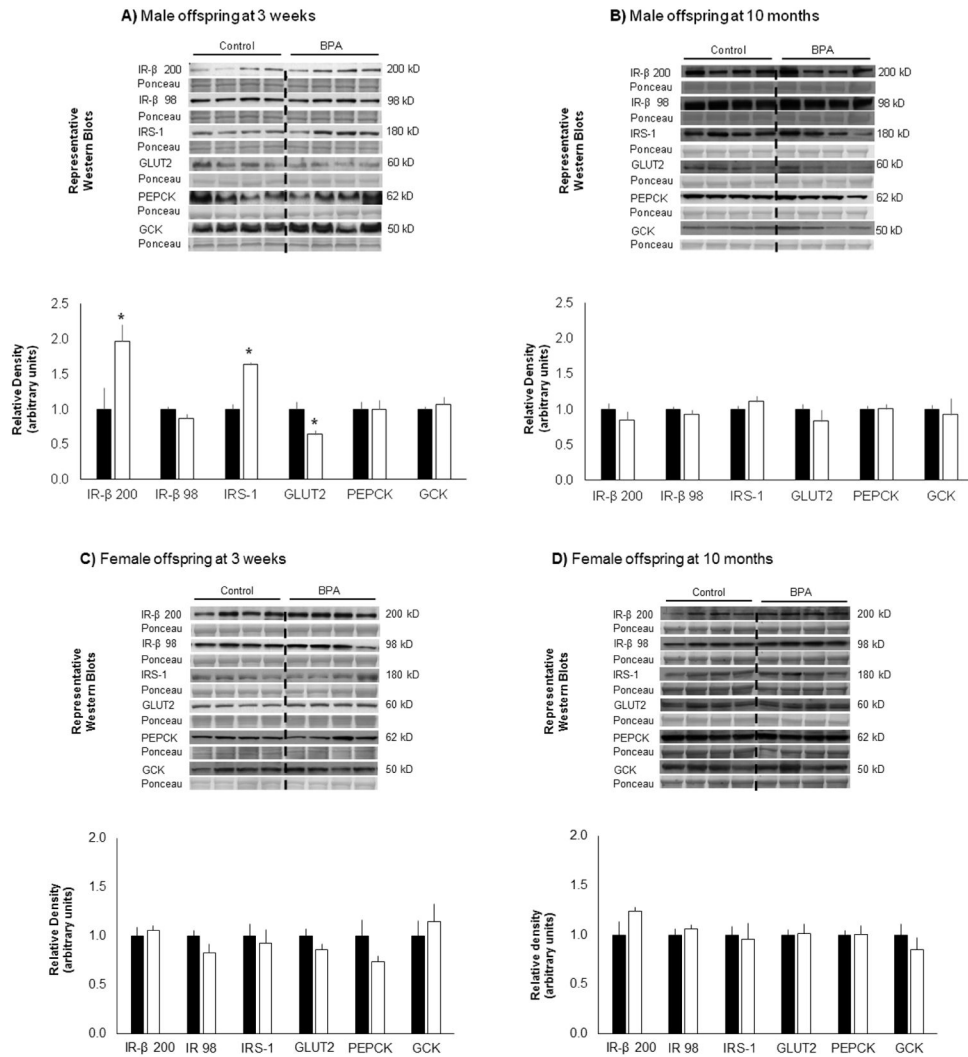


Figure 1C) 24 Weeks: Male Glucose Tolerance Test

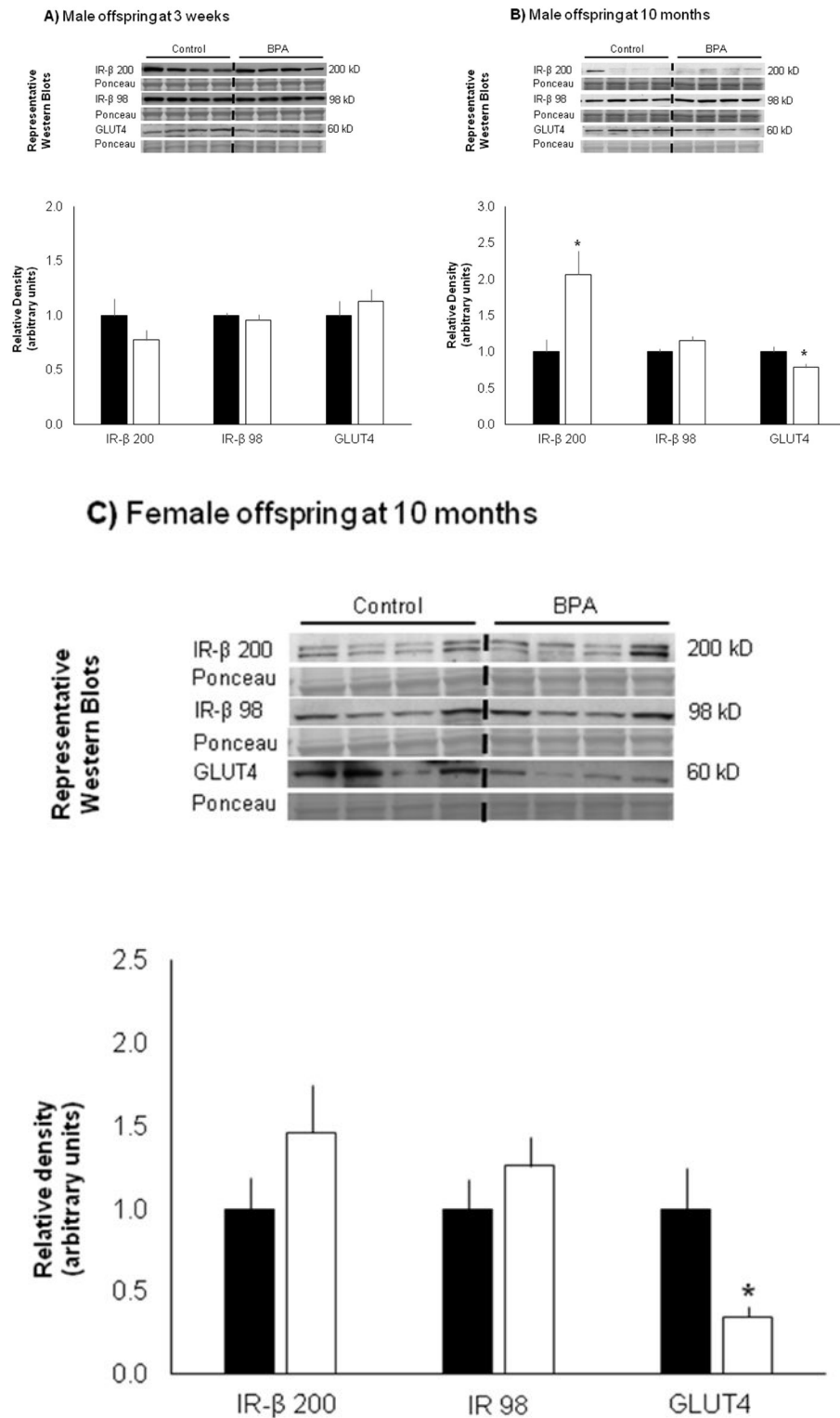


**Figure 1D) 24 Weeks: Female Glucose Tolerance Test****Figure 1.**

Values are mean  $\pm$  SEM in the blood concentration of glucose and the area under the glucose clearance curve (AUC) following glucose administration (1 mg/g body weight i.p. D-glucose at time = 0 min) in (A) male and (B) female control (solid symbols; n = 6) and BPA-treated (open symbols; n = 6) rat offspring at 6 weeks of postnatal age, and in (C) male and (D) female control (solid symbols; n = 5) and BPA-treated (open symbols; n = 5) rat offspring at 24 weeks of postnatal age. \*P < 0.05 *versus* control within the same sex; two-way repeated measures ANOVA plus Tukey test, with time and treatment as factors (glucose clearance curve) and Student's t test (area under the curve).



**Figure 2.** Representative Western Blots and quantitative analysis of the protein abundance of the insulin receptor beta (IR-β; 200 kD precursor molecule; 98 kD mature IR-β subunit), the insulin receptor substrate 1 (IRS-1), the glucose transporter 2 (GLUT2) and the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucokinase (GCK) in liver tissue from male control (solid bars; n = 5) and BPA-treated (open bars; n = 5) rat offspring at (A) 3 weeks and (B) 10 months of postnatal age and (C) female control (solid bars; n = 5) and BPA-treated (open bars; n = 5) rat offspring at 3 weeks and (D) 10 months of postnatal age. Ponceau S staining served as a loading control. Values are mean ± SEM. \*P < 0.05 versus control within the same immunoblot; Student's t test.



**Figure 3.** Representative Western Blots and quantitative analysis of the protein abundance of the insulin receptor beta (IR-β) and the glucose transporter 4 (GLUT4) in skeletal muscle from



male control (solid bars; n = 5) and BPA-treated (open bars; n = 5) rat offspring at (A) 3 weeks and (B) 10 months of postnatal age and female control (solid bars; n = 5) and BPA-treated (open bars; n = 5) rat offspring at (C) 10 months of postnatal age. Ponceau S staining served as a loading control. Values are mean  $\pm$  SEM. \*P < 0.05 *versus* control within the same immunoblot; Student's t test.

Table 1

## Blood Glucose and Plasma Insulin Concentrations

Concentrations of blood glucose and plasma insulin during the glucose tolerance test (GTT) (1 mg/g body weight i.p. D-glucose at time = 0 min), and the ratio of insulin to glucose in male and female offspring of control and BPA-treated rat dams from birth to 24 weeks of age. Values are mean  $\pm$  s.e.m.

Age	Variable	Time of data collection	Male		Female	
			Control	BPA	Control	BPA
3 Weeks						
	Basal blood glucose (mg/dl)	At tissue collection	124 $\pm$ 5	121 $\pm$ 12	122 $\pm$ 5	101 $\pm$ 10
	Basal plasma insulin (ng/ml)	At tissue collection	0.19 $\pm$ 0.01	0.31 $\pm$ 0.13	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01
	Insulin:glucose ratio	At tissue collection	1.5 $\pm$ 0.2	2.3 $\pm$ 0.7	1.5 $\pm$ 0.2	1.9 $\pm$ 0.2
6 Weeks						
	Basal plasma glucose (mg/dl)	GTT 0 min	80 $\pm$ 7	77 $\pm$ 7	88 $\pm$ 4	86 $\pm$ 6
	Basal plasma insulin (ng/ml)	GTT 0 min	0.18 $\pm$ 0.01	0.20 $\pm$ 0.01	0.20 $\pm$ 0.01	0.21 $\pm$ 0.01
	Insulin:glucose ratio	GTT 0 min	2.7 $\pm$ 0.5	2.5 $\pm$ 0.3	2.3 $\pm$ 0.2	2.4 $\pm$ 0.2
24 Weeks						
	Basal plasma glucose (mg/dl)	GTT 0 min	87 $\pm$ 2	102 $\pm$ 2	102 $\pm$ 4	92 $\pm$ 4
	Basal plasma insulin (ng/ml)	GTT 0 min	0.60 $\pm$ 0.08	1.02 $\pm$ 0.49*	0.40 $\pm$ 0.03	0.58 $\pm$ 0.11
	Insulin:glucose ratio	GTT 0 min	6.8 $\pm$ 0.8	9.98 $\pm$ 4.67	4.0 $\pm$ 0.4	6.28 $\pm$ 1.05*
	Plasma glucose (mg/dl)	GTT 15 min	147 $\pm$ 6	154 $\pm$ 8	139 $\pm$ 7	125 $\pm$ 11
	Plasma insulin (ng/ml)	GTT 15 min	1.46 $\pm$ 0.25	2.98 $\pm$ 0.42*	0.51 $\pm$ 0.05	1.47 $\pm$ 0.46*
	Insulin:glucose ratio	GTT 15 min	9.7 $\pm$ 1.3	19.5 $\pm$ 2.7*	3.7 $\pm$ 0.5	11.7 $\pm$ 3.3*

\* P<0.05 versus control within the same sex. Data were analysed by two-way ANOVA with sex and treatment as factors.